Science of the Total Environment 660 (2019) 260–268

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Effects of four years of elevated ozone on microbial biomass and extracellular enzyme activities in a semi-natural grassland

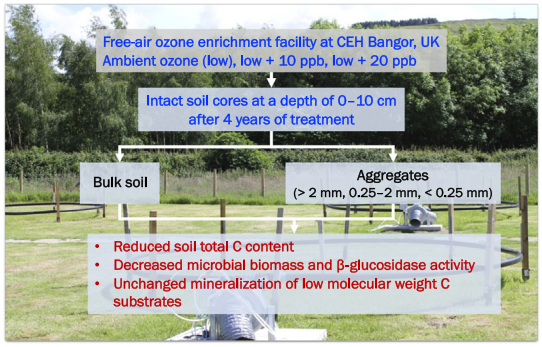
Jinyang Wang [a,*], Felicity Hayes [b], Robert Turner [a], David R. Chadwick [a], Gina Mills [b], Davey L. Jones [a,c]

[a] School of Natural Sciences, Environment Centre Wales, Bangor University, Bangor, Gwynedd LL57 2UW, UK
[b] Centre for Ecology and Hydrology, Environment Centre Wales, Bangor, Gwynedd LL57 2UW, UK
[c] UWA School of Agriculture and Environment, University of Western Australia, Crawley, WA 6009, Australia

HIGHLIGHTS

- A temperate grassland was exposed to ozone for 4 years under field conditions.
- Soil total carbon and β-glucosidase activity were decreased under elevated ozone.
- Neither ozone nor its interaction with aggregate size affected microbial activities.
- Elevated ozone could alter the size and activity of soil microbial community.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 October 2018
Received in revised form 4 January 2019
Accepted 5 January 2019
Available online 7 January 2019

Editor: Yasutomo Hoshika

Keywords:
(semi-)Natural vegetation
Climate change
Hydrolytic enzymes
FACE
Soil aggregates

ABSTRACT

Reduced belowground carbon (C) allocation by plants exposed to ozone may change properties and activities of the microbial community in soils. To investigate how soil microbial biomass and extracellular enzyme activities respond to elevated ozone, we collected soils from a temperate grassland after four years of ozone exposure under fully open-air field conditions. We measured soil microbial biomass, the metabolism of low molecular weight C substrates and hydrolytic extracellular enzyme activities in both bulk soil and isolated aggregates to assess changes in microbial activity and community function. After four years of elevated ozone treatment, soil total organic C was reduced by an average of 20% compared with ambient condition. Elevated ozone resulted in a small but insignificant reduction (4–10%) in microbial biomass in both bulk soil and isolated aggregates. Activities of extracellular enzymes were generally not affected by elevated ozone, except β-glucosidase, whose activity in bulk soil was significantly lower under elevated ozone than ambient condition. Activities of β-glucosidase, leucine aminopeptidase and acid phosphatase were higher in microaggregates (<0.25 mm) as compared to macroaggregates (>0.25 mm). Elevated ozone had no effects on mineralization rates of low molecular weight C substrates in both bulk soil and isolated aggregates. We therefore conclude that the size and activity rather than function of the soil microbial community in this semi-natural grassland are altered by elevated ozone.

1. Introduction

Tropospheric ozone is currently considered to be a key air pollutant because of its negative impact on plant productivity in most parts of the

* Corresponding author.
E-mail address: jyw217@gmail.com (J. Wang).

https://doi.org/10.1016/j.scitotenv.2019.01.040
0048-9697/

world (Ashmore, 2005; Fuhrer, 2009). During the past three decades, the background concentration of tropospheric ozone over the Northern Hemisphere midlatitudes has increased at a rate of 0.5–2% per year (Vingarzan, 2004). Further increases in the Northern Hemisphere background ozone concentrations may occur over this century if current emission trends continue (Meehl et al., 2007), although this view is being questioned (Oltmans et al., 2013; Ridley et al., 2017). Studies exploring ecosystem responses to elevated ozone have received widespread attention in the last two decades. There is mounting evidence that increasing tropospheric ozone concentration has many direct effects on plants, including lower net primary productivity (Ainsworth, 2008; Feng et al., 2008; Mills et al., 2018; Morgan et al., 2003), changes in plant chemistry (Booker et al., 2005; Kasurinen et al., 2007; Morgan et al., 2003), reduced stomatal conductance of plants (Feng et al., 2008; VanLoocke et al., 2012; Wittig et al., 2007), reduced root growth (Grantz et al., 2006), as well as altered root longevity and turnover (Andersen, 2003).

In contrast to the aboveground part, belowground processes in soils in response to elevated ozone have received less attention, despite its critical roles in biogeochemical cycles (Agathokleous et al., 2016; Andersen, 2003; Fuhrer et al., 2016). Since the penetration of ozone into the soil is limited (Toet et al., 2009), the indirect effects of ozone exposure on belowground communities and ecosystem processes are primarily due to reduced C allocation below ground. The belowground components (e.g. soil microorganisms) responses to elevated ozone in terrestrial ecosystems occur indirectly through plant-derived deposits, which has not been well documented. Under fully open-air field conditions or in open-top chambers, for example, how the composition and structure of the soil microbial community respond to elevated ozone has been examined in a soybean field (He et al., 2014), a wheat field (Li et al., 2013), a subarctic forest (Kasurinen et al., 2005), a temperate forest (Phillips et al., 2002) and a hay meadow (Kanerva et al., 2008). However, the results in these studies are conflicting, showing that elevated ozone altered (He et al., 2014; Kanerva et al., 2008; Kasurinen et al., 2005; Phillips et al., 2002) or had no significant effect (Li et al., 2013) on the composition and structure of the soil microbial community. Thus, while the inconsistent findings have often been attributed to the differences in experimental durations and other factors (e.g. fumigation facility, ecosystem type and management regime), this reflects an incomplete understanding of the response of soil microorganisms to elevated ozone.

Soil microorganisms are the main sources of crucial enzymes in the cycling of main nutrients (e.g. C, N and P). Moreover, soil enzyme activities are highly sensitive to environmental changes and could serve as indicators of various changes in the plant-soil system (Burns et al., 2013; Saiya-Cork et al., 2002). Activities of extracellular enzymes are strongly regulated by the presence of plants, and the release of labile substrates by living roots into soil enhances extracellular enzyme activities (Nannipieri et al., 2002). Therefore, the aforementioned changes in belowground plant growth under elevated ozone could have the potential to alter both substrate availability and extracellular enzyme activities (Andersen, 2003). Studies in aspen and aspen-birch forest ecosystems have shown that elevated ozone significantly reduced cellobiohydrolase activity but did not affect *N*-acetyl-glucosaminidase activity in the forest floor after 2- or 10-year treatment (Edwards and Zak, 2011; Larson et al., 2002). In a lysimeter study with young planted beech, Esperschütz et al. (2009) reported that soil extracellular enzyme activities were generally not affected after 4 years of ozone treatment. In contrast, Williamson et al. (2010) measured the decomposition rates of wetland plants exposed to elevated ozone and showed that the responses of activities of β-glucosidase and *N*-acetyl-glucosaminidase to elevated ozone were species-dependent. Thus, how soil extracellular enzyme activities respond to elevated ozone remains uncertain.

Soil aggregation physically protects certain soil organic matter (SOM) fractions via influencing soil microbial communities and activities. In general, soil aggregates are fractionated by three different approaches: wet-sieving (Six et al., 1998), dry-sieving (Chenu and Cosentino, 2011) and optimal moisture (Dorodnikov et al., 2009; Kristiansen et al., 2006). To link in situ microbial communities and activities with ecological processes, the optimal moisture approach can provide an advantage of minimizing microbial responses to lab processing for a wide-range of biological assays (i.e., microbial biomass and extracellular enzyme activities) (Bach and Hofmockel, 2014). The reported decrease of the available substrates under elevated ozone, through decreased C allocation and fluxes into belowground components, are expected to affect microbial biomass and extracellular enzyme activities (Andersen, 2003). However, less is known about how extracellular enzyme activities respond to elevated ozone in either bulk soil or isolated aggregates.

In this study, we aimed (i) to investigate changes in soil properties, microbial biomass and extracellular enzyme activities in bulk soil after four years of elevated ozone treatment, and (ii) to relate these changes observed in bulk soil to contrasting environment of differently sized aggregates. Given the aforementioned ozone effects on above- and belowground components, we hypothesized that field experimental exposure to elevated ozone in a grassland ecosystem would change soil microbial biomass and extracellular enzyme activities. For verifying this hypothesis, we collected soils from a temperate, semi-natural grassland after four years of ozone treatment under fully open-air field conditions.

2. Material and methods

2.1. Experiment site

Soil samples were taken from the ozone free-air controlled exposure (O_3-FACE) field located at CEH Bangor Air Pollution Facility, Abergwyngregyn, North Wales, UK (13 m asl, 53°15′N, 4°01′W). The study site has a temperate oceanic climate, with a mean annual soil temperature of 11 °C at 10 cm depth and a mean annual rainfall of 1250 mm. The soil is classified as Eutric Cambisol (FAO) or Dystric Eutrudepts (US Soil Taxonomy) with a sandy clay loam texture, which is derived from Ordovician postglacial alluvial deposits. Vegetation was classified as *Lolium perenne leys* and related grasslands according to the UK National Vegetation Classification (MG7; Rodwell, 1992), without sheep grazing for >15 years prior to this study. No fertilizer was applied at this site throughout the experimental period. Grass was cut 2–3 times during each growing season.

The O_3-FACE system was established in the spring of 2014, consisting of nine rings of 4 m diameter. Three ozone treatments with three replicates, namely low (ambient air), medium (ambient air + 10 ppb) and high (ambient air + 20 ppb), were randomly assigned to the rings (Table 1), where the latter two treatments are hereafter referred to as elevated ozone. The rings were arranged in a replicated 3×3 Latin square with 10 m between the centers of each ring. Ozone was generated by passing oxygen from a SeQual Integra 10 Oxygen Concentrator (SeQual Technologies, Inc., San Diego, CA, USA) through a Pacific Ozone G11 ozone generator (Benicia, California, USA). Small fans (Redring Xpelair Group Ltd., Southampton, UK) were used to push the ozone through the delivery pipe (65 mm, with 3 mm holes every 10 cm). Ozone delivery was achieved via computer controlled (LabView Version 2012, National Instruments) solenoid valves operating using pulse width modulation. Wind speed was monitored continuously (WindSonic, Gill Instruments Ltd., UK) and used to instantaneously adjust solenoid operation and thus ozone delivery. Ozone release was reduced at wind speeds below 16 m s^{-1} and did not occur when wind speeds fell below 2 m s^{-1}. Ozone was sampled adjacent to the plants in each ring at a height of 30 cm for approximately 3.5 min in every 30-min using an ozone analyzer (Thermo-Scientific, Model 49i, Reading, UK). Compared with previous studies using similar free-air systems (Paoletti et al., 2017; Watanabe et al., 2013), at very high wind speeds the ozone concentrations may not be well controlled and thus did not reach the target maximum concentrations. Despite this, we still got

Table 1
Mean ozone concentrations (24 h), mean daily maximum ozone concentration and AOT40 in daylight hours (08:00 to 20:00 GMT) measured in the ozone free-air controlled exposure (O_3-FACE) experiment at CEH Bangor Air Pollution Facility during the growing seasons in 2014–2017. Values represent means ± SEM (n = 3).

	July–October 2014			May–September 2015			June–September 2016			May–October 2017		
Ozone level	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)	Mean conc. (ppb)	Daily max. (ppb)	AOT40 (ppm h)
Low	28.2 ± 1.2	39.9 ± 1.4	1.1 ± 0.2	28.1 ± 0.4	40.5 ± 0.5	3.5 ± 0.5	20.6 ± 0.1	31.8 ± 0.8	0.4 ± 0.0	22.9 ± 0.6	32.8 ± 0.5	0.3 ± 0.0
Medium	36.8 ± 4.0	68.1 ± 11.1	7.1 ± 3.1	40.5 ± 5.4	71.4 ± 15.9	8.4 ± 2.2	43.3 ± 3.6	77.4 ± 6.6	20.3 ± 4.8	44.1 ± 2.5	86.1 ± 4.2	20.2 ± 4.7
High	49.5 ± 5.8	99.9 ± 12.5	16.2 ± 5.2	40.4 ± 1.6	67.8 ± 2.9	11.2 ± 1.1	62.6 ± 7.7	101.5 ± 11.2	46.2 ± 10.3	54.9 ± 6.1	106.5 ± 12.3	31.9 ± 10.1

elevated ozone with the higher in the 'high' ozone treatment compared to that of the 'medium' treatment as the solenoid valves were <1 m from the O_3-FACE rings, the response time of ozone delivery to track windspeed was fast. Exposure to elevated ozone lasted from 17 July to 13 October in 2014, from 13 May to 11 September in 2015, from 1 June to 30 September in 2016, and from 25 May to 9 October in 2017. Ozone release was 93, 67, 93 and 99% of the time during the fumigation periods in the years 2014, 2015, 2016, and 2017, respectively.

2.2. Aggregate-size fractionation

Soil was collected from the top 10 cm of soil using 6.5 cm-diameter soil cores in November 2017. Three intact soil cores were collected from each ring, placed in CO_2 permeable polythene bags and then transported to the laboratory. Each soil core was gently broken up along natural points of weakness and passed through an 8-mm sieve, removing visible roots and rocks. Replicated soil cores were combined into one composite sample for each ring and then stored at 4 °C to await further analysis. Prior to aggregate-size fractionation, subsamples of bulk soil were obtained from the cold-dried soils. Similar to previous studies (Bach and Hofmockel, 2014; Kristiansen et al., 2006), the optimal moisture approach was used for aggregate isolation to minimize microbial responses to lab processing for the following biological assays. Briefly, soils were cold dried at 4 °C to approximately 10% gravimetric water content. Approximately 400 g of cold-dried soil was placed on a stack of sieves including 2 mm- and 0.25 mm-mesh openings. The stack was bolted to a circular sieve shaker intend for soil particle analysis and shaken at 200–250 rpm for 3 min. Soil was gently removed from each sieve and weighed to determine the mass distribution of aggregates into the following fractions: large macroaggregates (>2 mm), small macroaggregates (0.25–2 mm) and microaggregates (<0.25 mm). Subsamples of bulk soil and individual aggregate-size fractions were saved to determine gravimetric water content, total C, microbial biomass and mineralization rates of low molecular weight C substrates. Subsamples for the enzyme assay detailed below were frozen immediately at −20 °C until analysis.

2.3. Soil analysis

Bulk density was determined after insertion of 100 cm^3 metal rings into the soil, removal of soil, and drying at 105 °C (24 h). Bulk density was calculated by dividing soil mass by core volume. Soil characteristics of both bulk soil and aggregate fractions were determined. Soil water content was determined gravimetrically by drying soil at 105 °C (24 h). Soil pH was measured using standard electrodes in a 1:2.5 (w/v) soil-to-deionized water mixture. Subsamples of bulk soil and aggregate fractions were directly extracted with 0.5 M K_2SO_4 (1:5 w/v) for available soil C and N pools measurement. For soil microbial biomass, additional subsamples were fumigated for 24 h with chloroform and similarly extracted with 0.5 M K_2SO_4 (1:5 w/v) (Vance et al., 1987). The 0.5 M K_2SO_4 extracts of non-fumigation and fumigation samples were quantified using a Multi N/C 2100 TOC analyzer (AnalytikJena, Jena, Germany) to determine soil dissolved organic C (DOC), microbial biomass C and N. Microbial biomass C and N concentrations were corrected using correction factors of 0.45 for C and 0.54 for N (Brookes et al., 1985; Wu et al., 1990). Total C (TC) and N (TN) of oven-dried and ground soils were determined with a TruSpec® elemental analyzer (Leco Corp., St Joseph, MI, USA). Based on the relative weight distribution of aggregates, the total microbial biomass C in different aggregates were recalculated for bulk soil. Net N mineralization and nitrification rates were determined by the aerobic incubation of soil samples for 14 days at 10 °C in the dark (Hart et al., 1994), followed by extraction with 0.5 M K_2SO_4 and analyzing for soil mineral N as described above.

Carbon mineralization was estimated using a short-term incubation method following Robertson et al. (1999). Briefly, 20 g fresh soils for bulk soil and aggregate fractions was moistened to field moisture content (25%) with deionized water in a 1-L jar. The mason jar was closed with airtight screw-cap lid, fitted with a gas sampling port (butyl rubber septum) at the center, and was incubated at 10 °C for 21 d. Soil respiration were measured on 1, 3, 5, 7, 14 and 21 d after incubation by measuring CO_2 concentration in the headspace air samples of the jar using a portable infrared gas analyzer (EGM-5 Environmental Gas Monitor for CO_2, PP Systems, Hitchin, UK). Carbon mineralization rate was calculated and expressed as mg C kg^{-1} h^{-1}.

In addition, the mineralization of glucose, amino acids and peptide were determined to estimate rates of low molecular weight dissolved organic C and N following the method of Hill et al. (2012). Briefly, 1 g fresh weight (equivalent to *c.* 0.87 g dry weight) soil was placed into a 1.5-mL microcentrifuge tube in which a hole had been pierced in bottom. This assembly was placed into another intact microcentrifuge tube. To the surface of the soil, 150 μL ^{14}C-labelled glucose (25 μM, 1.85 kBq mL^{-1}), amino acids (10 μM, 1.55 kBq mL^{-1}) and peptide (10 μM of L-trialanine, 1 kBq mL^{-1}) were added. It has been suggested that an incubation period of 3 min can reflect maximum variance between treatments (Hill et al., 2012). Thus, these samples were incubated at 20 °C for 3 min and then centrifuged at 4000 *g* for 1 min to facilitate collection of free soil solution. An aliquot of this solution was then transferred to a 6-mL scintillation vial to which 4 mL Scintisafe3 Scintillation cocktail (Fisher Scientific, Loughborough, Leicestershire, UK) was added before analysis using a Wallac 1404 liquid scintillation counter (Wallac, EG&G, Milton Keynes, UK). The amino acids consisted of an equimolar mix of 20 different L-amino acids (glycine, isoleucine, arginine, glutamine, phenylalanine, histidine, asparagine, valine, threonine, leucine, alanine, methionine, cysteine, lysine, tryptophan, serine, proline, glutamate, aspartic acid and ornithine).

2.4. Enzyme assays

The potential activities of six extracellular hydrolytic enzymes: β-glucosidase, cellobiohydrolase, β-xylosidase, *N*-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase were measured according to the fluorimetric protocol of Saiya-Cork et al. (2002) with modification by DeForest (2009). Briefly, 1 g of fresh soils was suspended in 125 mL sodium acetate buffer with pH adjusted to mean of soils. Soil suspensions were pipetted into 96-well microplates, and enzyme activities were determined by adding 4-methylumbelliferyl (MUB)- or 7-amino-4-methylcoumarin (AMC)-linked substrates for a final concentration of 40 μM. Assays were incubated in the dark for 2 h, and the reactions were stopped with 10 μL 0.5 M NaOH. The microplates were then scanned on a fluorescence spectrophotometer (Cary Eclipse,

Agilent Technologies, Inc., Santa Clara, CA, USA) using the excitation and emission filters at 365 and 450 nm, respectively. Potential enzyme activity for bulk soil and aggregate-size fractions was expressed as MUB or AMC released in nanomol per gram of dry soil or aggregate and hour (nmol g^{-1} soil h^{-1} or nmol g^{-1} aggregate h^{-1}) as described previously (DeForest, 2009). Specific activities of extracellular enzymes were also calculated as a measure of activity per unit microbial biomass and expressed as MUB or AMC released in nanomol per milligram microbial biomass C and hour (nmol mg^{-1} C_{mic} h^{-1}). The recovery of potential enzyme activity was calculated and expressed as a proportion of the bulk soil based on the weight distribution of aggregates.

2.5. Statistical analysis

All data were checked for assumptions of normality and log-transformed if necessary. A linear mixed effect model (LME, package LME4; Bates et al., 2014) was used to test ozone and/or aggregate-size class effects on investigated parameters with column and row included as random effects. Multiple comparisons between treatment means were conducted using post-hoc Tukey HSD tests (glht package: 'multcomp'). We accepted P values of $P \leq 0.05$ as significant and those with $P > 0.05$, but <0.1 as marginally significant. All statistical analyses were performed in R version 3.2.2 (R Development Core Team, 2015).

3. Results

3.1. The O_3-FACE system

The semi-natural grassland was exposed to ozone under fully open-air field conditions from July 17, 2014 through to October 9, 2017 during the growing season, with an average of 101 days effective fumigation. Inter-annual variations in ambient ozone concentration (24 h means) showed only a small variation and ranged from 20.6 ppb in 2016 to 28.2 ppb in 2014 (Table 1). Across all years, mean ozone concentrations in medium and high ozone rings were 69 and 116% higher than that in ambient air, respectively. Accumulated exposures above a threshold of 40 ppb (AOT40) averaged 1.3 ± 0.7 ppm h in the ambient rings, 14.0 ± 3.6 ppm h in the medium ozone rings and 26.4 ± 8.0 ppm h in the high ozone rings over the four-year period.

3.2. Soil properties, low molecular weight C substrate mineralization and enzyme activities in bulk soils

After 4 years of ozone treatment, soil total C and N were lower by an average of 20% and 16% under elevated ozone (medium and high ozone rings) than ambient ozone, respectively (both $P < 0.05$; Table 2), while soil bulk density, pH and C-to-N ratio did not differ between treatments. There was an apparent decrease in DOC and microbial biomass C in the elevated ozone treatments, which was not statistically significant when compared with those of the ambient ozone treatment. The ratios of microbial biomass C to total C were higher in the elevated ozone treatments than the ambient treatment ($P = 0.06$). Neither short-term C mineralization nor mineralization of low molecular weight C substrates for bulk soil was affected by elevated ozone.

Averaged over all treatments, higher extracellular enzyme activities in bulk soil were found for β-glucosidase and acid phosphatase (on average 293 and 578 nmol g^{-1} soil h^{-1}, respectively), while the other four enzymes showed lower and similar activities (Table 2). Elevated ozone significantly decreased β-glucosidase activity ($P < 0.05$) but not the activities of cellobiohydrolase, β-xylosidase, *N*-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase in bulk soil.

3.3. Aggregate-size distribution, total C and microbial biomass C content

Elevated ozone did not affect the relative distribution of three aggregate fractions (Table 3). Large and small macroaggregates dominated in this grassland soil, whereas the microaggregate fraction accounted for a very small percentage of total soil mass ($P < 0.001$). The weight distribution among the aggregate-size classes of the bulk soil was as follows: large macroaggregates (>2 mm) contributed 52.1–57.4%, small macroaggregates (0.25–2 mm) 35.6–38.3% and microaggregates (<0.25 mm) 6.5–9.6% of the weight of bulk soil. Total C content were higher in the large macro- and microaggregate fractions than in the small macroaggregate fraction ($P < 0.001$) but did not significantly differ between ozone treatments within each aggregate fraction.

Across aggregate fractions, microbial biomass C showed a marginally significant reduction by an average of 10% under elevated ozone ($P = 0.086$; Table 3). There was no clear relationship between microbial biomass C and aggregate-size classes. Relative to the bulk soil, the total microbial biomass C in different aggregates showed approximately 100% recoveries across ozone treatments (Fig. 1A). The ratios of microbial

Table 2
Soil characteristics, mineralization rates of low molecular weight C substrates and potential extracellular enzyme activity under different ozone treatments.

	Ozone level			
	Low	Medium	High	*P* value
Total C (g C kg^{-1})	39.8 ± 1.7	31.3 ± 0.4	32.6 ± 2.2	*
Total N (g N kg^{-1})	3.5 ± 0.2	2.8 ± 0.1	3.0 ± 0.2	*
C:N ratio	11.6 ± 0.9	11.2 ± 0.3	10.8 ± 0.4	*NS*
Bulk density (g cm^{-3})	0.83 ± 0.01	0.83 ± 0.02	0.87 ± 0.01	*NS*
pH	5.1 ± 0.2	5.3 ± 0.1	5.1 ± 0.1	*NS*
Dissolved organic C (mg C kg^{-1})	215 ± 11	192 ± 12	202 ± 12	*NS*
Microbial biomass C (mg C kg^{-1})	903 ± 37	889 ± 33	849 ± 51	*NS*
Microbial biomass N (mg N kg^{-1})	95 ± 3	107 ± 6	96 ± 10	*NS*
Microbial biomass C-to-N ratio	9.5 ± 0.5	8.4 ± 0.2	8.9 ± 0.4	*NS*
Microbial biomass C-to-total C ratio (%)	2.27 ± 0.08	2.84 ± 0.08	2.61 ± 0.17	•
C mineralization (mg C kg^{-1} h^{-1})	1.13 ± 0.13	0.75 ± 0.08	1.18 ± 0.29	*NS*
Glucose mineralization (mg C kg^{-1} h^{-1})	1.27 ± 0.12	1.32 ± 0.26	1.34 ± 0.10	*NS*
Amino acids mineralization (mg N kg^{-1} h^{-1})	0.15 ± 0.01	0.18 ± 0.02	0.17 ± 0.00	*NS*
Peptide mineralization (mg N kg^{-1} h^{-1})	0.31 ± 0.02	0.30 ± 0.04	0.30 ± 0.02	*NS*
β-glucosidase (nmol g^{-1} soil h^{-1})	332 ± 28	293 ± 20	224 ± 11	*
Cellobiohydrolase (nmol g^{-1} soil h^{-1})	50.5 ± 10.7	74.0 ± 10.7	54.4 ± 15.5	*NS*
N-acetyl-glucosaminidase (nmol g^{-1} soil h^{-1})	40.7 ± 3.1	47.7 ± 9.2	53.0 ± 0.6	*NS*
β-xylosidase (nmol g^{-1} soil h^{-1})	39.0 ± 3.8	47.1 ± 4.3	39.8 ± 8.1	*NS*
Leucine aminopeptidase (nmol g^{-1} soil h^{-1})	19.5 ± 0.9	23.7 ± 3.4	20.1 ± 1.1	*NS*
Acid phosphatase (nmol g^{-1} soil h^{-1})	537 ± 34	535 ± 36	599 ± 108	*NS*

Values represent means ± SEM (n = 3). Statistical results from linear mixed effect model with ozone as a fixed factor and column/row as random effects are reported. *NS*, • and * indicate not significant ($P \geq 0.1$), significant difference at $P < 0.1$ and $P < 0.05$, respectively.

Table 3
Aggregate-size distribution, organic C content and microbial biomass C in soil aggregates under different ozone treatments.

	Weights distribution (%)			C content (g C kg^{-1})			Microbial biomass C (mg C kg^{-1})		
Aggregate-size class	Low	Medium	High	Low	Medium	High	Low	Medium	High
>2 mm	57.4 ± 3.2	56.6 ± 1.6	52.1 ± 1.4	41.0 ± 2.4	36.8 ± 2.1	35.9 ± 1.0	912 ± 47	829 ± 24	891 ± 43
0.25–2 mm	36.1 ± 2.5	35.6 ± 2.1	38.3 ± 1.9	32.0 ± 2.8	31.8 ± 0.4	28.8 ± 0.2	896 ± 9	935 ± 5	848 ± 86
<0.25 mm	6.5 ± 0.9	7.8 ± 0.5	9.6 ± 0.4	37.1 ± 1.6	36.5 ± 1.6	36.8 ± 1.3	1160 ± 92	958 ± 65	819 ± 34
Ozone	*NS*			*NS*			•		
Aggregate size	***			***			*NS*		
Interaction	*NS*			*NS*			•		

Values represent means ± SEM (n = 3). Statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row as random effects are reported. *NS*, • and *** indicate not significant ($P \geq 0.1$), significant difference at $P < 0.1$ and $P < 0.001$, respectively.

biomass C to total C were affected by aggregate-size class ($P < 0.01$) and its interaction with ozone ($P = 0.064$; Fig. 1B).

3.4. Low molecular weight C substrate mineralization and enzyme activities in isolated aggregates

As with bulk soil, short-term C mineralization in isolated aggregates did not differ between ozone treatments (Fig. 1C), though C mineralization rates in small macroaggregates and microaggregates were lower by 32 and 31%, respectively under elevated ozone as compared to ambient conditions. Neither ozone nor its interaction with aggregate-size class had effects on mineralization rates of low molecular weight C substrates, except that stimulated glucose mineralization was detected in the large macroaggregate from the high ozone treatment (Fig. 1D–F). It should be noted that the pronounced effects of aggregate-size class on mineralization rates of low molecular weight C substrates were primarily due to underestimated turnover in the large macroaggregates with a 3-min incubation period.

Activities of β-glucosidase, *N*-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase were distributed differently through aggregate-size classes ($P < 0.05$–0.01; Fig. 2). Across ozone treatments, activities of β-glucosidase and acid phosphatase were of the order microaggregate > large macroaggregate > small macroaggregate. The lowest activity of leucine aminopeptidase was found both in the high ozone treatment and small macroaggregate fraction. Activities of cellobiohydrolase and β-xylosidase showed similar across all aggregate-size classes irrespective of ozone. Since aggregate-size class had no effect on microbial biomass, the patterns of specific activities of extracellular enzymes are almost identical to patterns as seen above (data not shown). Cumulative proportional enzyme activity in isolated

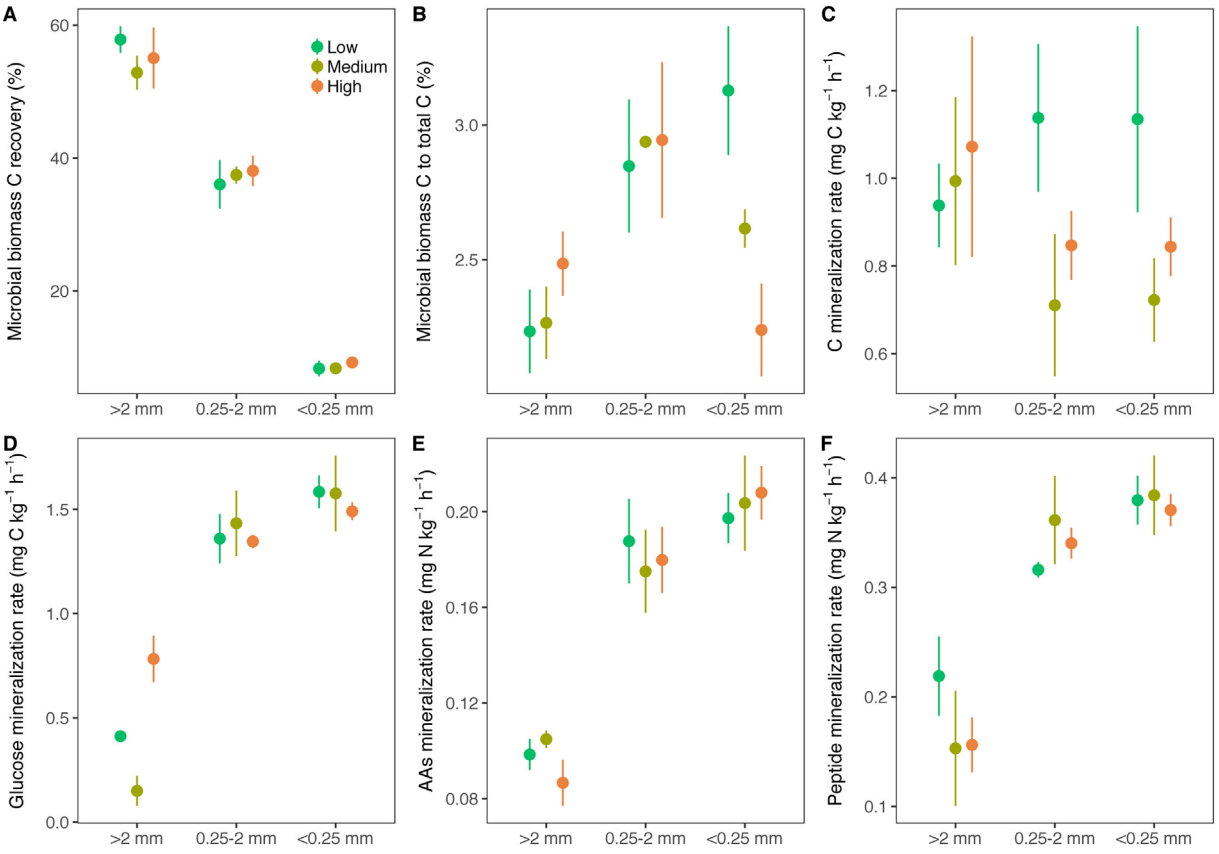


Fig. 1. Microbial biomass C recovery, microbial biomass C-to-total C ratio, short-term C mineralization, mineralization rates of low molecular weight C substrates (glucose, amino acids (AAs) and peptide) in three aggregate fractions under different ozone treatments. Values represent means ± SEM (n = 3). See text for further explanation on statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row as random effects.

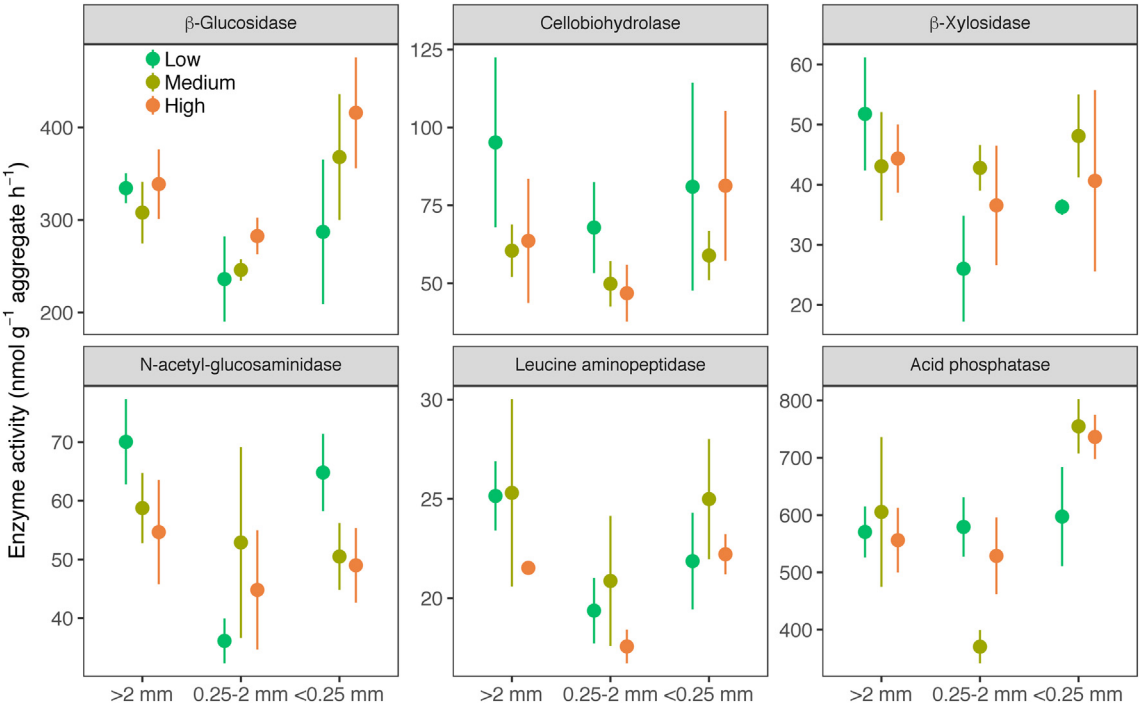


Fig. 2. Potential activities of β-glucosidase, cellobiohydrolase, β-xylosidase, *N*-acetyl-glucosaminidase, leucine aminopeptidase and acid phosphatase in three aggregate fractions under different ozone treatments. Values represent means ± SEM (n = 3). See text for further explanation on statistical results from linear mixed effect model with ozone and aggregate-size class as fixed factors and column/row as random effects.

aggregates did not differ from bulk soil, with somewhat larger variation ranged from 89% to 144% across enzymes (data not shown).

4. Discussion

4.1. Aggregate-size fractionation

According to the concept of aggregate hierarchy (Tisdall and Oades, 1982), the bulk soil has been fractionated into its constituent aggregates using different disruptive techniques (Chenu and Cosentino, 2011; Mendes et al., 1999; Six et al., 1998). In this study, we chose the optimal moisture sieving technique which allows limited mechanical stress to breakdown of macroaggregates along the planes of weakness, releasing the microaggregates located on surfaces of macroaggregates and along their planes of weakness (Dorodnikov et al., 2009; Kristiansen et al., 2006). The small portion of microaggregates isolated in this study (6.5–9.6%) was comparable to those reported in other studies (Bach and Hofmockel, 2016; Kumar et al., 2017). This finding further supports the claim that free microaggregates and the microaggregates adhering on the surface of macroaggregates are isolated. On the other hand, the most distinguishing characteristic of optimal moisture sieving compared to the conventional wet- and dry sievings is to minimize effects on the soil microbial community and biological parameters. This is supported by our results showing that cumulative recoveries of microbial biomass and enzyme activity were 99–102% and 89–144%, respectively, across all treatments and enzymes.

The aggregate weight distribution detected here were in the order: large macroaggregates > small macroaggregates > microaggregates (Table 3). This is in agreement with other studies showing that large and small macroaggregates dominated in agricultural soils (Bach and Hofmockel, 2014; Kristiansen et al., 2006; Kumar et al., 2017). The distribution of aggregate-size classes was not altered after four years of ozone treatment, although a significant reduction of root biomass under elevated ozone was detected (ambient ozone: 1176 ± 142 g m^{-2} vs. elevated ozone: 725 ± 87 g m^{-2}; $P = 0.024$). Consistent with this finding, the high plant density resulted in a two-fold increase of root biomass but had no effect on aggregate redistribution in a maize field (Kumar et al., 2017). Consequently, our findings indicate that elevated ozone had no effect on the distribution of soil aggregate-size classes, although there are negative impacts of elevated ozone on root growth and belowground C allocation (Andersen, 2003; Grantz et al., 2006).

4.2. Effects of elevated ozone on microbial biomass in bulk soil and isolated aggregates

Numerous studies have been conducted to assess the effect of elevated ozone on soil microbial biomass, but the results remain controversial. Whereas some studies showed a decrease in microbial biomass (Bao et al., 2015; Kanerva et al., 2008; Phillips et al., 2002), others reported no difference (Cheng et al., 2011; Zhang et al., 2014) or even an increased microbial biomass (Mörsky et al., 2008) from soils under elevated ozone. Our results support those studies that found a negative response of soil microbial biomass to elevated ozone, partly corroborating our initial hypothesis. Ozone exposure is considered to alter C flux to soil via changes in rhizodeposition and litter quality or quantity (Andersen, 2003), and therefore, the decreased microbial biomass in bulk soil is most likely due to reduced root biomass and substrate availability under elevated ozone. Further, this is primarily associated with a significant reduction of microbial biomass in the microaggregate fraction under elevated vs. ambient ozone (Table 3). Since macroaggregates and microaggregates are inhabited predominately by fungal and bacterial communities, respectively, we speculate that bacterial communities in microaggregates might be strongly affected by elevated ozone in this grassland soil. In contrast, some studies have shown that elevated ozone significantly reduced both fungal biomass and the fungal-to-bacterial ratio, suggesting that fungi may be more sensitive to elevated ozone as compared to bacteria (Kanerva et al., 2008; Li et al., 2013; Phillips et al., 2002). This inconsistency could be due to the differences in ecosystem types, experimental duration and methods, as well as environmental conditions. Nonetheless, we are aware that the present study is the first to assess the response of

microbial biomass to elevated ozone among different aggregate fractions and further investigations are required.

The lack of correlation between soil microbial biomass and aggregate-size class contradicts the findings of others in agricultural soils, where they found soil microbial biomass were positively or negatively correlated with decreasing aggregate size (Dorodnikov et al., 2009; Kumar et al., 2017). Different microbial biomass between microaggregates and macroaggregates are often attributed to the contrasting environment of differently sized aggregates, which in turn contributes to the differential distribution of bacteria and fungi in micro- and macroaggregates (Chenu et al., 2001; Gupta and Germida, 1988; Jastrow et al., 2007). Since the composition and structure of the soil microbial community were not determined in isolated aggregates, we are not sure if the lack of correlation between microbial biomass and aggregate-size class is related to changes in microbial communities. In a recent review, Gupta and Germida (2015) also point out that further studies are warranted to investigate the distribution and temporal dynamics of microbes in distinct aggregates. While total organic C and microbial biomass C did not differ between ozone treatments within each aggregate fraction, the reduced ratio of microbial biomass C to total organic C in microaggregates may have contributed to the decline in total C in bulk soil under elevated ozone (Sparling, 1992). In contrast, the increased ratio of microbial biomass C to total organic C in the bulk soil under elevated ozone may be caused by decreases in total organic C content rather than microbial biomass.

4.3. Effects of elevated ozone on extracellular enzyme activities in bulk soil and isolated aggregates

As an overall indicator of microbial activity, the significantly lower activity of β-glucosidase in bulk soil under elevated vs. ambient ozone supports the findings suggesting depressed microbial activity due to reduced C allocation into the belowground ecosystem (Andersen, 2003). Further, the significant reduction of the ratio of the natural logarithm of β-glucosidase and the sum of *N*-acetyl-glucosaminidase and leucine aminopeptidase in bulk soil indicates that elevated ozone could stimulate microbes to produce enzymes towards acquisitions of organic N (Sinsabaugh et al., 2008), despite the absence of ozone effect on individual enzymes (Table 2). Chitin is one of the dominant sources of organic N to soil, and *N*-acetyl-glucosaminidase releases small, N-containing amino sugars from chitin in addition to C (Olander and Vitousek, 2000). In this grassland without fertilizers application and grazing for a long-term period, elevated ozone might have resulted in microbially decomposing recalcitrant organic matter for both energy source and nutrient demand (e.g. N). Thus, these findings support our hypothesis regarding ozone effects on extracellular enzyme activities. Yet, there are very few studies addressing the responses of extracellular enzyme activity to elevated ozone and showing mixed results. For example, studies in aspen and aspen-birch forest ecosystems reported that elevated ozone had no effects on enzyme activities in the second year of treatment (Larson et al., 2002), whereas after 10 years cellobiohydrolase activity was affected in the forest floor but *N*-acetyl-glucosaminidase remained unaffected (Edwards and Zak, 2011). Further, Williamson et al. (2010) measured the decomposition rates of wetland plants exposed to elevated ozone and concluded that the response of hydrolytic enzyme activity to ozone was species dependent. Collectively, these conflicting results indicates that ozone effects on extracellular enzymes remain poorly understood and further work is needed.

Across all enzymes, enzyme activities were somewhat higher in microaggregates than in macroaggregates irrespective of ozone treatment. This is consistent with the previous findings showing that the highest enzyme activities occurred in microaggregates, especially for β-glucosidase (Dorodnikov et al., 2009; Kumar et al., 2017). We found that enzyme activities in isolated aggregates generally equaled or exceeded those in bulk soil and may have been even greater if there were enzyme losses during the aggregate fractionation. This supports the findings by several researchers who reported similar or higher recovery of enzyme activity in isolated aggregates as compared to the bulk soil (Allison and Jastrow, 2006; Bach and Hofmockel, 2014; Dorodnikov et al., 2009). This indicates that a lack of enzyme activity might be not responsible for C accumulation associated with soil aggregation. In addition, elevated ozone affected neither enzyme activities nor low molecular weight C substrate mineralization within each aggregate fraction, suggesting that substrate utilization patterns of soil microbial communities were unchanged.

5. Conclusions

To our knowledge the present study is the first to assess the responses of microbial biomass and extracellular enzyme activities in bulk soil and isolated aggregates to elevated ozone under O_3-FACE conditions. Our results demonstrated that elevated ozone for a period of four years had negative impacts on both soil C sequestration and total microbial biomass activity (i.e., decreased microbial biomass and β-glucosidase activity), which was mainly due to reduced belowground C allocation. Ozone exposure did not affect soil aggregation in this semi-natural grassland, probably contributing to the absence of effects of ozone and its interaction with aggregate-size class on low molecular weight C substrate utilization and extracellular enzyme activities. It should also be noted that the small, statistically insignificant changes (e.g. microbial biomass) could be associated with high variability. Therefore, our results suggest that changes in the quantity and quality of plant C inputs at elevated ozone can contribute to reduce soil total C content but not to alter the function of the soil microbial community in this semi-natural grassland.

Acknowledgements

This work was supported by the European Commission under Horizon 2020 for a Marie Skłodowska-Curie Actions COFUND Fellowship (663830-BU-048) and by the Welsh Government and Higher Education Funding Council for Wales through the Sêr Cymru National Research Network for Low Carbon, Energy and Environment. We thank Aled Williams for technical support in running the O_3-FACE facility. We thank Dr. Elena Paoletti and two anonymous reviewers for helpful comments in the revision of the manuscript.

References

Agathokleous, E., Saitanis, C.J., Wang, X., Watanabe, M., Koike, T., 2016. A review study on past 40 years of research on effects of tropospheric O_3 on belowground structure, functioning, and processes of trees: a linkage with potential ecological implications. Water Air Soil Pollut. 227. https://doi.org/10.1007/s11270-015-2715-9.

Ainsworth, E.A., 2008. Rice production in a changing climate: a meta-analysis of responses to elevated carbon dioxide and elevated ozone concentration. Glob. Chang. Biol. 14, 1642–1650. https://doi.org/10.1111/j.1365-2486.2008.01594.x.

Allison, S.D., Jastrow, J.D., 2006. Activities of extracellular enzymes in physically isolated fractions of restored grassland soils. Soil Biol. Biochem. 38, 3245–3256. https://doi.org/10.1016/j.soilbio.2006.04.011.

Andersen, C.P., 2003. Source-sink balance and carbon allocation below ground in plants exposed to ozone. New Phytol. 157, 213–228. https://doi.org/10.1046/j.1469-8137.2003.00674.x.

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell Environ. 28, 949–964. https://doi.org/10.1111/j.1365-3040.2005.01341.x.

Bach, E.M., Hofmockel, K.S., 2014. Soil aggregate isolation method affects measures of intra-aggregate extracellular enzyme activity. Soil Biol. Biochem. 69, 54–62. https://doi.org/10.1016/j.soilbio.2013.10.033.

Bach, E.M., Hofmockel, K.S., 2016. A time for every season: soil aggregate turnover stimulates decomposition and reduces carbon loss in grasslands managed for bioenergy. GCB Bioenergy 8, 588–599. https://doi.org/10.1111/gcbb.12267.

Bao, X., Yu, J., Liang, W., Lu, C., Zhu, J., Li, Q., 2015. The interactive effects of elevated ozone and wheat cultivars on soil microbial community composition and metabolic diversity. Appl. Soil Ecol. 87, 11–18. https://doi.org/10.1016/j.apsoil.2014.11.003.

Bates, D., Mächler, M., Bolker, B., Walker, S., 2014. Fitting Linear Mixed-effects Models Using lme4 (arXiv Prepr. arXiv1406.5823).

Booker, F.L., Prior, S.A., Torbert, H.A., Fiscus, E.L., Pursley, W.A., Hu, S., 2005. Decomposition of soybean grown under elevated concentrations of CO_2 and O_3. Glob. Chang. Biol. 11, 685–698. https://doi.org/10.1111/j.1365-2486.2005.00939.x.

Brookes, P.C., Landman, A., Pruden, G., Jenkinson, D.S., 1985. Chloroform fumigation and the release of soil nitrogen: a rapid direct extraction method to measure microbial biomass nitrogen in soil. Soil Biol. Biochem. 17, 837–842. https://doi.org/10.1016/0038-0717(85)90144-0.

Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein, M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: current knowledge and future directions. Soil Biol. Biochem. 58, 216–234. https://doi.org/10.1016/j.soilbio.2012.11.009.

Cheng, L., Booker, F.L., Burkey, K.O., Tu, C., da Shew, H.D., Rufty, T.W., Fiscus, E.L., Deforest, J.L., Hu, S., 2011. Soil microbial responses to elevated CO_2 and O_3 in a nitrogen-aggrading agroecosystem. PLoS One 6. https://doi.org/10.1371/journal.pone.0021377.

Chenu, C., Cosentino, D., 2011. Microbial regulation of soil structural dynamics. The Architecture and Biology of Soils: Life in Inner Space. CABI Wallingford, UK, pp. 37–70.

Chenu, C., Hassink, J., Bloem, J., 2001. Short-term changes in the spatial distribution of microorganisms in soil aggregates as affected by glucose addition. Biol. Fertil. Soils 34, 349–356. https://doi.org/10.1007/s003740100419.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biol. Biochem. 41, 1180–1186. https://doi.org/10.1016/j.soilbio.2009.02.029.

Dorodnikov, M., Blagodatskaya, E., Blagodatsky, S., Marhan, S., Fangmeier, A., Kuzyakov, Y., 2009. Stimulation of microbial extracellular enzyme activities by elevated CO_2 depends on soil aggregate size. Glob. Chang. Biol. 15, 1603–1614. https://doi.org/10.1111/j.1365-2486.2009.01844.x.

Edwards, I.P., Zak, D.R., 2011. Fungal community composition and function after long-term exposure of northern forests to elevated atmospheric CO_2 and tropospheric O_3. Glob. Chang. Biol. 17, 2184–2195. https://doi.org/10.1111/j.1365-2486.2010.02376.x.

Esperschütz, J., Pritsch, K., Gattinger, A., Welzl, G., Haesler, F., Buegger, F., Winkler, J.B., Munch, J.C., Schloter, M., 2009. Influence of chronic ozone stress on carbon translocation pattern into rhizosphere microbial communities of beech trees (*Fagus sylvatica* L.) during a growing season. Plant Soil 323, 85–95. https://doi.org/10.1007/s11104-009-0090-2.

Feng, Z., Kobayashi, K., Ainsworth, E.A., 2008. Impact of elevated ozone concentration on growth, physiology, and yield of wheat (*Triticum aestivum* L.): a meta-analysis. Glob. Chang. Biol. 14, 2696–2708. https://doi.org/10.1111/j.1365-2486.2008.01673.x.

Fuhrer, J., 2009. Ozone risk for crops and pastures in present and future climates. Naturwissenschaften 96, 173–194. https://doi.org/10.1007/s00114-008-0468-7.

Fuhrer, J., Val Martin, M., Mills, G., Heald, C.L., Harmens, H., Hayes, F., Sharps, K., Bender, J., Ashmore, M.R., 2016. Current and future ozone risks to global terrestrial biodiversity and ecosystem processes. Ecol. Evol. 6, 8785–8799. https://doi.org/10.1002/ece3.2568.

Grantz, D.A., Gunn, S., Vu, H.B., 2006. O_3 impacts on plant development: a meta-analysis of root/shoot allocation and growth. Plant Cell Environ. 29, 1193–1209. https://doi.org/10.1111/j.1365-3040.2006.01521.x.

Gupta, V.V.S.R., Germida, J.J., 1988. Distribution of microbial biomass and its activity in different soil aggregate size classes as affected by cultivation. Soil Biol. Biochem. 20, 777–786. https://doi.org/10.1016/0038-0717(88)90082-X.

Gupta, V.V.S.R., Germida, J.J., 2015. Soil aggregation: influence on microbial biomass and implications for biological processes. Soil Biol. Biochem. 80, A3–A9. https://doi.org/10.1016/j.soilbio.2014.09.002.

Hart, S.C., Stark, J.M., Davidson, E.A., Firestone, M.K., 1994. Nitrogen mineralization, immobilization, and nitrification. Methods Soil Anal. Part 2. Microbiol. Biochem. Prop., pp. 985–1018 https://doi.org/10.2136/sssabookser5.2.c42

He, Z., Xiong, J., Kent, A.D., Deng, Y., Xue, K., Wang, G., Wu, L., Van Nostrand, J.D., Zhou, J., 2014. Distinct responses of soil microbial communities to elevated CO_2 and O_3 in a soybean agro-ecosystem. ISME J. 8, 714–726. https://doi.org/10.1038/ismej.2013.177.

Hill, P.W., Farrell, M., Jones, D.L., 2012. Bigger may be better in soil N cycling: does rapid acquisition of small L-peptides by soil microbes dominate fluxes of protein-derived N in soil? Soil Biol. Biochem. 48, 106–112. https://doi.org/10.1016/j.soilbio.2012.01.023.

Jastrow, J.D., Amonette, J.E., Bailey, V.L., 2007. Mechanisms controlling soil carbon turnover and their potential application for enhancing carbon sequestration. Clim. Chang. 80, 5–23. https://doi.org/10.1007/s10584-006-9178-3.

Kanerva, T., Palojärvi, A., Rämö, K., Manninen, S., 2008. Changes in soil microbial community structure under elevated tropospheric O_3 and CO_2. Soil Biol. Biochem. 40, 2502–2510. https://doi.org/10.1016/j.soilbio.2008.06.007.

Kasurinen, A., Keinänen, M.M., Kaipainen, S., Nilsson, L.O., Vapaavuori, E., Kontro, M.H., Holopainen, T., 2005. Below-ground responses of silver birch trees exposed to elevated CO_2 and O_3 levels during three growing seasons. Glob. Chang. Biol. 11, 1167–1179. https://doi.org/10.1111/j.1365-2486.2005.00970.x.

Kasurinen, A., Peltonen, P.A., Julkunen-Tiitto, R., Vapaavuori, E., Nuutinen, V., Holopainen, T., Holopainen, J.K., 2007. Effects of elevated CO_2 and O_3 on leaf litter phenolics and subsequent performance of litter-feeding soil macrofauna. Plant Soil 292, 25–43. https://doi.org/10.1007/s11104-007-9199-3.

Kristiansen, S.M., Schjønning, P., Thomsen, I.K., Olesen, J.E., Kristensen, K., Christensen, B.T., 2006. Similarity of differently sized macro-aggregates in arable soils of different texture. Geoderma 137, 147–154. https://doi.org/10.1016/j.geoderma.2006.08.005.

Kumar, A., Dorodnikov, M., Splettstößer, T., Kuzyakov, Y., Pausch, J., 2017. Effects of maize roots on aggregate stability and enzyme activities in soil. Geoderma 306, 50–57. https://doi.org/10.1016/j.geoderma.2017.07.007.

Larson, J.L., Zak, D.R., Sinsabaugh, R.L., 2002. Extracellular enzyme activity beneath temperate trees growing under elevated carbon dioxide and ozone. Soil Sci. Soc. Am. J. 66, 1848. https://doi.org/10.2136/sssaj2002.1848.

Li, X., Deng, Y., Li, Q., Lu, C., Wang, J., Zhang, H., Zhu, J., Zhou, J., He, Z., 2013. Shifts of functional gene representation in wheat rhizosphere microbial communities under elevated ozone. ISME J. 7, 660–671. https://doi.org/10.1038/ismej.2012.120.

Meehl, G.A., Stocker, T.F., Collins, W.D., Friedlingstein, P., Gaye, T., Gregory, J.M., Kitoh, A., Knutti, R., Murphy, J.M., Noda, A., Raper, S.C.B., Watterson, I.G., Weaver, A.J., Zhao, Z.C., 2007. Global climate projections. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.), IPCC, 2007: Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, pp. 747–846.

Mendes, I.C., Bandick, A.K., Dick, R.P., Bottomley, P.J., 1999. Microbial biomass and activities in soil aggregates affected by winter cover crops. Soil Sci. Soc. Am. J. 63, 873–881.

Mills, G., Sharps, K., Simpson, D., Pleijel, H., Broberg, M., Uddling, J., Jaramillo, F., Davies, W.J., Dentener, F., Van den Berg, M., Agrawal, M., Agrawal, S.B., Ainsworth, E.A., Büker, P., Emberson, L., Feng, Z., Harmens, H., Hayes, F., Kobayashi, K., Paoletti, E., Van Dingenen, R., 2018. Ozone pollution will compromise efforts to increase global wheat production. Glob. Chang. Biol. 24, 3560–3574. https://doi.org/10.1111/gcb.14157.

Morgan, P.B., Ainsworth, E.A., Long, S.P., 2003. How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. Plant Cell Environ. 26, 1317–1328. https://doi.org/10.1046/j.0016-8025.2003.01056.x.

Mörsky, S.K., Haapala, J.K., Rinnan, R., Tiiva, P., Saarnio, S., Silvola, J., Holopainen, T., Martikainen, P.J., 2008. Long-term ozone effects on vegetation, microbial community and methane dynamics of boreal peatland microcosms in open-field conditions. Glob. Chang. Biol. 14, 1891–1903. https://doi.org/10.1111/j.1365-2486.2008.01615.x.

Nannipieri, P., Kandeler, E., Ruggiero, P., 2002. Enzyme activities and microbiological and biochemical processes in soil. In: Burns, R.G., Dick, R.P. (Eds.), Enzymes in the Environment: Activity, Ecology, and Applications. Marcel Dekker, New York, NY, pp. 1–33.

Olander, L.P., Vitousek, P.M., 2000. Regulation of soil phosphatase and chitinase activity by N and P availability. Biogeochemistry 49, 175–190. https://doi.org/10.1023/A:1006316117817.

Oltmans, S.J., Lefohn, A.S., Shadwick, D., Harris, J.M., Scheel, H.E., Galbally, I., Tarasick, D.W., Johnson, B.J., Brunke, E.G., Claude, H., Zeng, G., Nichol, S., Schmidlin, F., Davies, J., Cuevas, E., Redondas, A., Naoe, H., Nakano, T., Kawasato, T., 2013. Recent tropospheric ozone changes - a pattern dominated by slow or no growth. Atmos. Environ. 67, 331–351. https://doi.org/10.1016/j.atmosenv.2012.10.057.

Paoletti, E., Materassi, A., Fasano, G., Hoshika, Y., Carriero, G., Silaghi, D., Badea, O., 2017. A new-generation 3D ozone FACE (Free Air Controlled Exposure). Sci. Total Environ. 575, 1407–1414. https://doi.org/10.1016/j.scitotenv.2016.09.217.

Phillips, R.L., Zak, D.R., Holmes, W.E., White, D.C., 2002. Microbial community composition and function beneath temperate trees exposed to elevated atmospheric carbon dioxide and ozone. Oecologia 131, 236–244. https://doi.org/10.1007/s00442-002-0868-x.

R Development Core Team, 2015. R: A Language and Environment for Statistical Computing. Austria, Vienna.

Ridley, D.A., Cain, M., Methven, J., Arnold, S.R., 2017. Sensitivity of tropospheric ozone to chemical kinetic uncertainties in air masses influenced by anthropogenic and biomass burning emissions. Geophys. Res. Lett. 44, 7472–7481. https://doi.org/10.1002/2017GL073802.

Robertson, G.P., Wedin, D., Groffmann, P.M., Blair, J.M., Holland, E.A., Nadelhoffer, K.J., Harris, D., 1999. Soil carbon and nitrogen availability: nitrogen mineralization, nitrification, and soil respiration potentials. Standard Soil Methods for Long-Term Ecological Research. Oxford University Press, pp. 258–271.

Rodwell, J.S., 1992. British plant communities. Grasslands and Montane Communities. vol. 3. Cambridge University Press, Cambridge, UK.

Saiya-Cork, K., Sinsabaugh, R., Zak, D., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol. Biochem. 34, 1309–1315. https://doi.org/10.1016/S0038-0717(02)00074-3.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecol. Lett. 11, 1252–1264. https://doi.org/10.1111/j.1461-0248.2008.01245.x.

Six, J., Elliott, E.T., Paustian, K., Doran, J.W., 1998. Aggregation and soil organic matter accumulation in cultivated and native grassland soils. Soil Sci. Soc. Am. J. 62, 1367. https://doi.org/10.2136/sssaj1998.03615995006200050032x.

Sparling, G.P., 1992. Ratio of microbial biomass carbon to soil organic carbon as a sensitive indicator of changes in soil organic matter. Soil Res. 30, 195–207.

Tisdall, J.M., Oades, J.M., 1982. Organic matter and water-stable aggregates in soils. J. Soil Sci. 33, 141–163. https://doi.org/10.1111/j.1365-2389.1982.tb01755.x.

Toet, S., Subke, J.A., D'Haese, D., Ashmore, M.R., Emberson, L.D., Crossman, Z., Evershed, R.P., Barnes, J.D., Ineson, P., 2009. A new stable isotope approach identifies the fate of ozone in plant-soil systems. New Phytol. 182, 85–90. https://doi.org/10.1111/j.1469-8137.2009.02780.x.

Vance, E.D., Brookes, P.C., Jenkinson, D.S., 1987. An extraction method for measuring soil microbial biomass C. Soil Biol. Biochem. 19, 703–707. https://doi.org/10.1016/0038-0717(87)90052-6.

VanLoocke, A., Betzelberger, A.M., Ainsworth, E.A., Bernacchi, C.J., 2012. Rising ozone concentrations decrease soybean evapotranspiration and water use efficiency whilst increasing canopy temperature. New Phytol. 195, 164–171. https://doi.org/10.1111/j.1469-8137.2012.04152.x.

Vingarzan, R., 2004. A review of surface ozone background levels and trends. Atmos. Environ. 38, 3431–3442.

Watanabe, M., Hoshika, Y., Inada, N., Wang, X., Mao, Q., Koike, T., 2013. Photosynthetic traits of Siebold's beech and oak saplings grown under free air ozone exposure in northern Japan. Environ. Pollut. 174, 50–56. https://doi.org/10.1016/j.envpol.2012.11.006.

Williamson, J., Mills, G., Freeman, C., 2010. Species-specific effects of elevated ozone on wetland plants and decomposition processes. Environ. Pollut. 158, 1197–1206. https://doi.org/10.1016/j.envpol.2010.01.019.

Wittig, V.E., Ainsworth, E.A., Long, S.P., 2007. To what extent do current and projected increases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review of the last 3 decades of experiments. Plant Cell Environ. 30, 1150–1162. https://doi.org/10.1111/j.1365-3040.2007.01717.x.

Wu, J., Joergensen, R., Pommerening, B., Chaussod, R., Brookes, P.C., 1990. Measurement of soil microbial biomass C by fumigation-extraction—an automated procedure. Soil Biol. Biochem. 22, 1167–1169. https://doi.org/10.1016/0038-0717(90)90046-3.

Zhang, W., He, H., Li, Q., Lu, C., Zhang, X., Zhu, J., 2014. Soil microbial residue dynamics after 3-year elevated O_3 exposure are plant species-specific. Plant Soil 376, 139–149. https://doi.org/10.1007/s11104-013-1973-9.